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Transmittal of Provisional Patent Application for Filing
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I hereby certify that this Request, enclosed application and any other documents referred to as enclosed herein, are being deposited in an envelope with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date above and addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450.

Lynnea B. Anderson
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Request for Filing a Provisional Patent
Application Under 37 CFR §1.53(c)

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Sir:

This is a request for filing a Provisional Application for Patent under 37 CFR §1.53(c).

1. **Title of Invention:**

**METHODS FOR ISOLATION OF TRIPTOLIDE COMPOUNDS
FROM *Tripterygium Wilfordii***

2. **Inventor Applicant(s):**

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4. **Enclosed documents accompanying this transmittal sheet:**

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| <input checked="" type="checkbox"/> Return Post card for USPTO Date Stamp | <input checked="" type="checkbox"/> Cert. of Express Mail |
| <input checked="" type="checkbox"/> Specification Number of pages: <u>09</u> | <input checked="" type="checkbox"/> Drawings <u>00</u> sheets |
| <input checked="" type="checkbox"/> Claims Number of pages: <u>03</u> | <input checked="" type="checkbox"/> Abstract <u>01</u> page |

5. **Government Interest**

PROVISIONAL APPLICATION FILING ONLY

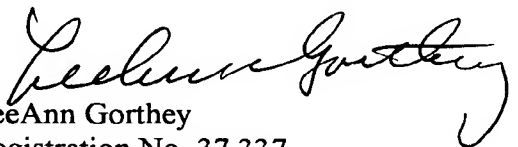
- ☐ This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government. The name of the U.S. Government Agency and the Government contract number are:

6. **Method of Payment:**

- ☒ Applicant claims small entity status. See 37 CFR §1.27
- ☒ Enclosed is a check in the amount of \$80.00 for the filing fee due. The Commissioner is hereby authorized to charge any deficiency in fees to Deposit Account 50-2207.

Respectfully submitted,

Date: Feb 9, 2004


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Methods for Isolation of Triptolide Compounds from *Tripterygium wilfordii***Field of the Invention**

5 The invention pertains to methods for extraction, isolation, and purification of triptolide and related molecules, such as triptolide and 16-hydroxytriptolide, from *Tripterygium wilfordii*.

References

- 10 Z. Cheng *et al.*, "Research on extraction technology of *Tripterygium*", *Chinese J. of Pharmaceuticals* **21**(10):435-436 (1990).
- S.M. Kupchan *et al.*, "Triptolide and triptolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*", *J. Am. Chem. Soc.* **94**(20):7194-7195 (1972).
- S.M. Kupchan *et al.*, U.S. Patent No. 4,005,108 (1977).
- P.E. Lipsky *et al.*, U.S. Patent No. 5,580,562 (Dec 1996).
- 15 K. Ren *et al.*, U.S. Appn. Pubn. No. 20040018260 (Jan 2004).
- T.T. Wiedmann *et al.*, U.S. Patent No. 5,843,452 (Dec 1998).
- C.P. Zhang *et al.*, "Studies on diterpenoids from leaves of *Tripterygium wilfordii*", *Acta Pharmaceutica Sinica* **28**(2):110-115 (1993).

20 **Background**

Compounds derived from the Chinese medicinal plant *Tripterygium wilfordii* (TW) have been identified as having useful therapeutic properties, particularly immunosuppressive activity and anticancer activity. These compounds include triptolide, triptolide and 16-hydroxy triptolide. Synthetic derivatives and prodrugs of

25 these compounds have also shown therapeutic activity, often in combination with improved pharmacological properties. See, for example, U.S. Patent Nos. 5,468,772 (Tripterinin compound and method), 5,648,376 (Immunosuppressant diterpene compound), 5,663,335 (Immunosuppressive compounds and methods), 5,759,550 (Method for suppressing xenograft rejection), 5,843,452 (Immunotherapy composition and method), 5,962,516 (Immunosuppressive compounds and methods), 6,150,539 (Triptolide prodrugs having high aqueous solubility), 6,294,546 (Uses of diterpenoid triepoxides as an antiproliferative agent), 6,537,984 (Uses of diterpenoid triepoxides as an antiproliferative agent), 6,548,537 (Triptolide prodrugs having high aqueous

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solubility), 6,569,893 (Amino acid derivatives of triptolide compounds as immune modulators and anticancer agents), 6,599,499 (Uses of diterpenoid triepoxides as an antiproliferative agent), and 6,620,843 (Anticancer treatment using triptolide prodrugs), each of which is incorporated herein by reference.

5 Isolation of the native compounds from the plant material has, to date, typically required laborious extraction and purification procedures. Kupchan *et al.* (1972, 1977) describe a method in which the root material is extracted with ethanol, the solid extract is dissolved in ethyl acetate and partitioned with water, and the ethyl acetate fraction is chromatographed on silica gel. Cheng *et al.* (1993) describe a method in which the first
10 extraction employs hot water, followed by addition of ethanol, filtration; removal of the ethanol, partitioning with chloroform, and chromatography on silica gel. The method described by Lipsky *et al.* (1996) employs subsequent extractions with chloroform, methanol, and toluene, with removal of solvent between each extraction, followed by chromatography on alumina and then on silica gel. Wiedmann *et al.* (1998) describe a
15 method in which the root material is extracted with refluxing aqueous ethanol, the solid extract is partitioned between dichloromethane and water, and the dichloromethane phase is concentrated and chromatographed on silica gel. Ken *et al.* (2004) describe a method in which the root is extracted repeatedly with ethanol, and the extracts are concentrated and extracted repeatedly with chloroform, followed by chromatographic
20 purification.

 In isolation methods to date, the initial extraction steps generally produce an extract which retains a large quantities of undesired materials, which then must be removed chromatographically, requiring large investments of time and materials. In view of the therapeutic utility of these compounds, higher efficiency methods for isolation and
25 purification are desired.

Summary of the Invention

 The invention provides an improved method of isolating triptolide and related compounds, *e.g.* triptolide and 16-hydroxytriptolide, from *Tripterygium wilfordii* (TW)
30 plant material. In accordance with the method, an extract of *Tripterygium wilfordii* plant material containing these compounds is formed and then purified. The extract is initially formed by

(a) extracting TW plant material, preferably root material, with aqueous ethanol, and concentrating to obtain a residue; and

(b) forming a slurry of this residue in an organic solvent, preferably a chlorinated hydrocarbon solvent, such as chloroform, methylene chloride, dichloroethane, or mixtures thereof; partitioning the slurry with water for a period of about 10 mins-10 hours; and then removing the water.

Typically, the extracting of step (a) includes three extractions with refluxing ethanol, each using 4-5 mL of ethanol per g of plant material, followed by pooling of the extracts; the slurry formed in step (b) comprises 8-12 volumes of organic solvent relative to the residue; and the partitioning of step (b) employs 1/2 to 2 volumes of water relative to the slurry.

The subsequent purification comprises the steps of:

further partitioning the slurry with an aqueous solution of base, removing the aqueous solution of base, and removing at least a portion of the organic solvent from the slurry;

washing the residue with a lipophilic solvent; and

eluting the residue from a silica gel adsorbent.

In one embodiment, this purification comprises, following steps (a) and (b) above:

(c) partitioning the slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a further residue;

(d) washing the further residue with a hydrocarbon solvent, to obtain a solid product; and

(e) purifying the solid product by silica gel chromatography.

In another embodiment, this purification comprises, following steps (a) and (b) above:

(c) partitioning the slurry of the residue with an aqueous solution of base, removing the aqueous solution, and removing a portion of the organic solvent, to obtain a concentrated slurry;

(d) adding silica gel to the concentrated slurry, in an amount effective to adsorb the triptolide and related compounds;

(e) washing the residue and silica gel with a hydrocarbon solvent; and

(f) eluting the triptolide and related compounds from the silica gel.

In a further embodiment, this purification comprises, following steps (a) and (b) above:

(c) removing the organic solvent from the slurry of the residue;

(d) washing the residue with a hydrocarbon solvent;

5 (e) forming a further slurry of the washed residue in an organic solvent selected from chloroform, methylene chloride, dichloroethane and mixtures thereof;

(f) partitioning the further slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a solid product; and

10 (g) purifying the solid product by silica gel chromatography.

In the aqueous solution of base, the base is preferably a water soluble hydroxide, carbonate or bicarbonate having a counterion selected from lithium, sodium, potassium, cesium, ammonium, and tetraalkylammonium, where alkyl is preferably C₁-C₄ alkyl. Suitable bases include, for example, NaOH, KOH, NaHCO₃, KHCO₃, Na₂CO₃ and
15 K₂CO₃. The solution may be selected, accordingly, from 0.1N - 2.5N aqueous NaOH, 0.1N - 2.5N aqueous KOH, 10% - 15% aqueous NaHCO₃, and 12% - 18% aqueous KHCO₃. The base partitioning is generally carried out for about 2 days, following a brief (e.g. 5-20 minutes, typically about 10 minutes) period of stirring. Optionally, following the removal of the aqueous solution of base, and prior to the removal of all or a portion
20 of the organic solvent, the organic solvent is washed with a dilute aqueous acidic solution.

The lipophilic solvent is preferably a hydrocarbon solvent selected from linear, branched and cyclic hydrocarbons having 5-7 carbon atoms, and mixtures thereof; examples include hexane and cyclohexane. In one embodiment, the hydrocarbon solvent
25 is hexane. The silica gel chromatography preferably employs a mobile phase comprising a nonpolar solvent, such as hexane, in combination with a more polar solvent, such as ethyl acetate.

Detailed Description of the Invention

30 I. Overview

The invention provides a procedure for purifying an extract of *Tripterygium wilfordii* (TW) plant material containing triptolide and related compounds, such as

tripdiolide and/or 16-hydroxytriptolide, and isolating these compounds. Compounds such as triptiolide, tripchloride, and triptonide may also be isolated.

As described below, the method includes treatment of an initial organic extract with aqueous base, which removes a significant amount of impurities at an early stage of the process, thereby increasing yield and reducing production costs. The base treatment effectively removes acidic or weakly acidic compounds (e.g. celastrol, triptoquinone A, triptoquinone G, 3-hydroxyoleanolic acid, polpunonic acid, tripterygic acid A, and phenolic compounds such as triptonoterpene, hypolide, triptophenolide, and triptonodial) from the organic TW extract. The base treatment also remove "oily" impurities from the extract via saponification and/or hydrolysis. This step has been found to remove about 70% of the undesired impurities from the extract, including oily materials whose removal typically generates a large majority of the cost of subsequent purification using traditional methods.

The method of the invention also includes an extraction with a lipophilic solvent, such as cyclohexane or a similar hydrocarbon-based solvent, e.g. hexanes, pentanes, petroleum ether, etc., to remove less polar impurities from the extract. This step further simplifies the subsequent chromatographic purification steps, by removing components which would otherwise typically be removed chromatographically.

20 II. Extraction Procedure

The *Tripterygium wilfordii* (TW) extract is initially formed by (i) extracting ground, chopped or otherwise finely divided TW plant material with aqueous ethanol, and concentrating the liquid extract to obtain a residue; (ii) forming a slurry of this residue in an organic solvent, preferably a chlorinated hydrocarbon solvent, typically selected from chloroform, methylene chloride, dichloroethane and mixtures thereof; (iii) partitioning the slurry with water for a period of about 10 mins-10 hours; and (iv) removing the water from the slurry.

The plant material may include the roots, stems, and leaves of *Tripterygium wilfordii*; preferably, the root material is used. The TW plant is found in the Fujiang Province and other southern provinces of China; TW plant material can generally be obtained in China or through commercial sources in the United States.

Preferably, the ethanol extraction (i) includes three extractions with refluxing ethanol, each using 4-5 mL of ethanol per gram of plant material, followed by pooling of the extracts. The amount of organic solvent used, typically chloroform or dichloroethane, is generally about 8-12 times the volume of the residue from step (i).

- 5 The partitioning step (iii) generally employs 1/2 to 2 volumes of water relative to the volume of slurry.

As used herein, "partitioning" of a mixture of two immiscible fluids generally refers to a short period of stirring, *e.g.* about 10-30 minutes, more typically 10-15 minutes, followed by settling of the mixture, typically for a period of hours or days. In this case,
10 the organic slurry and water are first stirred together, *i.e.* for about 10 minutes, and allowed to settle over a period of about 10 mins - 10 hours, preferably about 2 hours.

III. Purification Procedure

In accordance with the method of the invention, the slurry obtained following step
15 (iv) above is partitioned with an aqueous solution of base. In this process, the slurry and solution are first stirred together, *i.e.* for about 10 minutes, and allowed to settle over a period of about 10 mins-10 days, preferably about 1-4 days, more preferably about 2 days. The base is preferably a water soluble hydroxide, carbonate, or bicarbonate having a counterion selected from lithium, sodium, potassium, cesium, and ammonium.
20 Suitable bases include, for example, NaOH, KOH, NaHCO₃, KHCO₃, Na₂CO₃ and K₂CO₃. In preferred embodiments, the aqueous solution of base is selected from 0.1-2.5N aqueous NaOH, 0.1-2.5N aqueous KOH, 10%-15% aqueous NaHCO₃, and 12%-18% aqueous KHCO₃.

The aqueous solution of base is removed, and then, optionally, the organic solvent is
25 washed with a dilute aqueous acidic solution, *e.g.* 1% HCl. At least a portion of the organic solvent is then removed from the slurry.

The residue obtained, which may be substantially solid or a concentrated slurry, is then washed with a lipophilic solvent, followed by elution from a silica gel adsorbent. Preferably, the lipophilic solvent is a hydrocarbon solvent, preferably a saturated
30 hydrocarbon, selected from linear, branched and cyclic hydrocarbons having 5-7 carbon atoms, and mixtures thereof. In one embodiment, the solvent is cyclohexane.

Note that in variations of the procedure, as described below, the order of certain treatment steps may be altered.

Elution from a silica gel adsorbent (*i.e.* silica gel chromatography) preferably employs a solvent mixture, or mobile phase, comprising a non-polar solvent, such as a hydrocarbon, alkyl ether, or mixture thereof, in combination with a more polar solvent, such as an ester or ketone solvent. Such non-polar solvents include, for example, hexane, cyclohexane, petroleum ether, or THF. Such polar solvents include, for example, ethyl acetate, acetone, or methyl ethyl ketone (MEK). In one embodiment, the solvent mixture comprises cyclohexane and ethyl acetate. Solvent gradients may also be used, in accordance with known methods.

IV. Variations on the Purification Procedure

In one embodiment, substantially all of the organic solvent is removed from the slurry following removal of the aqueous base, to give a substantially solid residue. This residue is then washed with the lipophilic solvent to obtain a solid product, which is then purified by silica gel chromatography, as described above.

In another embodiment, only a portion of the organic solvent is removed from the slurry following removal of the aqueous base, to give a concentrated slurry. Silica gel is then added to the concentrated slurry, in an amount effective to adsorb triptolide and related compounds (*e.g.* triptolide and/or 16-hydroxytriptolide). The resulting mixture is then washed with the lipophilic solvent, and the triptolide and related compounds are then eluted from the silica gel.

In a further embodiment, extraction with the lipophilic solvent precedes the base treatment. Accordingly, prior to partitioning with base, the organic solvent is removed from the slurry obtained following step (iv) above, and the residue is washed with the lipophilic solvent. A further slurry of the washed residue is then formed, again in an organic solvent selected from chloroform, methylene chloride, dichloroethane and mixtures thereof, and this slurry is then partitioned with an aqueous solution of base, as described above, for a period of about 10 minutes to 10 days, preferably about 2 days. The aqueous base solution is removed, and substantially all of the organic solvent is then removed, to obtain a substantially solid residue, which is then purified by silica gel chromatography.

V. Exemplary Procedure

Following is an exemplary isolation procedure in accordance with one embodiment of the invention. This procedure is intended to illustrate and not to limit the invention.

A. Extraction

- 5 1. Dried TW biomass is ground into pieces (1 x 0.1 cm - 5 x 0.5 cm (length x diameter) for root core and stem; 0.1 – 2.0 cm in size (chip shape) for root bark). The ground TW biomass is refluxed with 50 – 95% (preferably 90%) ethanol for 2-5 (preferably 3) hours, 2-5 (preferably 3) times, at a weight/volume ratio of solid/ethanol of 1:4-6 (preferably 1:5) for the first
10 extraction and 1:3-5 (preferably 1: 4) for the subsequent extractions.
2. The extracts are pooled, and the is ethanol removed under reduced pressure to give a dark slurry.

B. Isolation (including base treatment and hydrocarbon extraction)

- 15 1. The slurry is suspended in 8-12, preferably 10, volumes of dichloroethane or chloroform.
2. Water is added, in an amount of 1/2-2 volumes, preferably 1/2 volume, to the suspension. The mixture is stirred for about 10 minutes and allowed to settle over a period of 1-10 hours, preferably 2 hours.
- 20 3. The water layer is removed, and ½-1 volume, preferably 1/2 volume, of 0.1-2.5N, preferably 0.5 N NaOH or KOH solution, or 10-15% NaHCO₃, is added to the organic phase. The mixture is stirred gently for about 10 minutes, then left for 1-10 days, preferably 4 days, to allow the layers to separate.
4. The aqueous phase is removed.
- 25 5. Water is added, in an amount of 1/4-1 volume, preferably 1/4 volume, relative to the organic phase. The mixture is stirred for about 10 minutes and left for 1-3 hours. Optionally, the mixture is washed twice at this stage with 1% HCl.
6. The aqueous phase is removed, and a drying agent, such as Na₂SO₄ or
30 MgSO₄ (3g/100mL), is added to the organic phase. The mixture is stirred and then filtered to remove the drying agent.
7. The organic solvent is removed completely under reduced pressure.

8. Cyclohexane is added to the resulting solid, and the mixture is stirred, *e.g.* for about 10 minutes, to suspend the solid.
9. The solid is removed by filtration and dried under reduced pressure at 40–60°C to obtain an intermediate product as a yellow powder.

5 C. Further Purification (silica gel chromatography)

1. The powder is dissolved in 1:1 cyclohexane:ethyl acetate at a concentration of 0.5-1.0 g/mL, preferably 0.75 g/mL.
2. The dissolved material is loaded onto a pre-equilibrated silica gel column (200-300 mesh, 100 x 1-20cm), using about 100g of silica gel per 1-3g, preferably per 2g, of the powder intermediate.
3. The product is eluted using the same solvent mixture at a flow rate of 10-30 ml/hr, preferably 18 ml/hour. Triptolide-enriched fractions are collected, monitoring with TLC or HPLC.
4. Triptolide-enriched fractions are pooled and the solvent removed.
- 15 5. Triptolide and/or related compounds are crystallized by temperature adjustment and/or solvent (*e.g.* acetone or ethyl ether) adjustment.
6. Optionally, column chromatography and/or crystallization are repeated.

CLAIMS

It is claimed:

- 5 1. A method for purifying an extract of *Tripterygium wilfordii* plant material containing triptolide and related compounds, wherein the extract is initially formed by
- (a) extracting plant material with aqueous ethanol, and concentrating to obtain a residue; and
- (b) forming a slurry of this residue in an chlorinated organic solvent; partitioning
- 10 the slurry with water for a period of about 10 mins-10 hours; and then removing the water;
- and wherein said purifying comprises the steps of:
- further partitioning the slurry with an aqueous solution of base, removing the aqueous solution of base, and removing at least a portion of the organic solvent from the
- 15 slurry;
- washing the residue with a lipophilic solvent; and
- eluting the residue from a silica gel adsorbent.
2. The method of claim 1, wherein said purifying comprises the steps of:
- 20 (c) partitioning the slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a further residue;
- (d) washing the further residue with a hydrocarbon solvent, to obtain a solid product; and
- (e) purifying the solid product by silica gel chromatography.
- 25 3. The method of claim 2, wherein a mobile phase comprising cyclohexane and ethyl acetate is used for said silica gel chromatography.
4. The method of claim 1, wherein said purifying comprises the steps of:
- 30 (c) partitioning the slurry of the residue with an aqueous solution of base, removing the aqueous solution, and removing a portion of the organic solvent, to obtain a concentrated slurry;

(d) adding silica gel to the concentrated slurry, in an amount effective to adsorb the triptolide and related compounds;

(e) washing the residue and silica gel with a hydrocarbon solvent; and

(f) eluting the triptolide and related compounds from the silica gel.

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5. The method of claim 1, wherein said purifying comprises the steps of:

(c) removing the organic solvent from the slurry of the residue;

(d) washing the residue with a hydrocarbon solvent;

(e) forming a further slurry of the washed residue in an organic solvent selected from chloroform, methylene chloride, dichloroethane and mixtures thereof;

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(f) partitioning the further slurry with an aqueous solution of base, then removing the aqueous solution, and then removing the organic solvent, to obtain a solid product; and

(g) purifying the solid product by silica gel chromatography.

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6. The method of claim 1, wherein the extracting of step (a) includes three extractions with refluxing ethanol, each using 4-5 mL of ethanol per g of plant material, followed by pooling of the extracts.

20 7. The method of claim 1, wherein the chlorinated organic solvent is selected from the group consisting of chloroform, methylene chloride, dichloroethane and mixtures thereof.

25 8. The method of claim 1, wherein the slurry formed in step (b) comprises 8-12 volumes of organic solvent relative to the residue, and the partitioning of step (b) employs 1/2 to 2 volumes of water relative to the slurry.

30 9. The method of claim 1, wherein the base is a water soluble hydroxide, carbonate or bicarbonate having a counterion selected from lithium, sodium, potassium, cesium, ammonium, and tetraalkylammonium.

10. The method of claim 9, wherein the base is selected from NaOH, KOH, NaHCO₃, KHCO₃, Na₂CO₃ and K₂CO₃.

11. The method of claim 10, wherein the aqueous solution of base is selected from 0.1N - 2.5N aqueous NaOH, 0.1N - 2.5N aqueous KOH, 10% - 15% aqueous NaHCO₃, and 12% - 18% aqueous KHCO₃.

12. The method of claim 1, wherein the partitioning with the aqueous solution of base is carried out for about 2 days.

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13. The method of claim 1, wherein, following the removing of the aqueous solution of base and prior to the removing of at least a portion of the organic solvent, the organic solvent is washed with a dilute aqueous acidic solution.

14. The method of claim 1, wherein the hydrocarbon solvent is selected from linear, branched and cyclic hydrocarbons having 5-7 carbon atoms, and mixtures thereof.

15. The method of claim 14, wherein the hydrocarbon solvent is cyclohexane.

16. The method of claim 1, wherein the plant material comprises root material.

17. The method of claim 1, wherein the related compounds comprise triptolide and/or 16-hydroxytriptolide.

ABSTRACT

Methods for extraction, isolation, and purification of therapeutically useful compounds from *Tripterygium wilfordii* are described. Extraction steps employing aqueous base and a hydrocarbon solvent, respectively, are found to increase the efficiency of the process and reduce the amount of material that must be removed by chromatography.